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Bacterial Symbionts Colonize the Accessory Nidamental Gland of the Squid *Loligo opalescens* via Horizontal Transmission

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Abstract. The accessory nidamental gland (AN gland), a reproductive organ of the mature female squid *Loligo opalescens*, harbors a dense culture of bacteria of unknown function. A multilayered sheath surrounding the *L. opalescens* egg case is similarly colonized by bacteria that presumably originate in the AN gland, as evidenced by their presence in the egg case at oviposition. This study investigates how these bacteria are transmitted to juvenile squid and examines some morphological consequences of bacterial colonization of AN gland tissues. By observing the structure of the AN gland in adults and the development and bacterial colonization of the gland in juveniles raised in captivity, we determined that the AN gland was absent in newly hatched squid and did not appear until 87 days post-hatching. At 129 days post-hatching, the organ displayed tubules composed of a single layer of epithelial cells and expressing numerous cilia and microvilli. These tubules were not yet fully formed and thus were open to the mantle cavity and external seawater, possibly to aid in the acquisition of microorganisms. Since the AN gland developed a considerable time after hatching, it most likely acquires its symbionts horizontally from environmental seawater and not vertically from the egg case sheath. The switch from expression of cilia to production of microvilli on the epithelial cell surface may dictate the competence of the tissue for bacterial colonization. Electron microscopic examination of juvenile and adult AN glands revealed that an analogous process occurs during the development of the related light organ of other cephalopod species that harbor symbiotic bacteria.

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Abbreviations: AN gland = accessory nidamental gland.

Introduction

When spawning, *Loligo opalescens* attaches its eggs to the sandy seafloor, building large masses of capsules that appear to be resistant to fungal, bacterial, and predator attack during their month-long embryonic development (Fields, 1965). Each egg capsule, containing about 200 embryos, is surrounded by a mucopolysaccharide sheath that disintegrates during the gestation period. Within the egg case, the embryos are embedded in gelatinous substances released from three accessory reproductive organs—the oviducal and nidamental glands, and presumably, the accessory nidamental (AN) gland (Arnold, 1984; Boletzky, 1986). This AN gland, in cephalopods such as *L. pealei* (Bloodgood, 1977), *Sepia officinalis* (Van den Branden *et al.*, 1980), and *L. forbesi* (Lum-Kong and Hastings, 1992), harbors a dense culture of symbiotic bacteria, suggesting that it may have a conserved function. Recently, a dense culture of bacteria was also described in layers of the egg capsule sheath in *L. opalescens* (Biggs and Epel, 1991). Because these bacteria were present in the egg capsules at oviposition, a transfer of bacteria from the AN gland of the spawning mother to the egg capsule was proposed. In addition, a protective role in defending the vulnerable embryos against microbial attack was postulated for these egg-capsule-associated bacteria. This

role is analogous to the anti-fungal protection provided by symbiotic bacteria associated with shrimp and lobster eggs (Gil-Turnes *et al.*, 1989; Gil-Turnes and Fenical, 1992).

This study addresses the primary events in the bacterial colonization of the naïve AN gland tissues after hatching of the juvenile squid. Bacteria from the egg sheath might be transmitted directly to the juvenile female at hatching ("vertical transmission"). Alternatively, transmission of bacteria from the water column might occur during or after hatching ("horizontal transmission").

An ultrastructural analysis was conducted to observe AN gland development and bacterial colonization in newly hatched juveniles, older juveniles raised in captivity, and glands from mature females. Our results indicate that the newly hatched squid do not contain a differentiated AN gland, and thus vertical transmission cannot occur. Rather, when the gland appears many weeks post-hatching, it is probably infected horizontally with bacteria from the water column.

Electron microscopic studies indicate that the bacterial colonization occurs in epithelial cell layers that have differentiated structures such as ciliated epithelium to facilitate inoculation. This host tissue competence is similar to that described for the *Euprymna scolopes-Vibrio fischeri* symbiosis in which the light organ of the squid becomes infected with bacteria (Montgomery and McFall-Ngai, 1994).

Materials and Methods

Squid culture

Adult specimens of *L. opalescens* Berry were captured live in Monterey Bay. Fresh egg capsules spawned by the captive squid were collected as previously described (Biggs and Epel, 1991). To obtain freshly hatched juvenile squid, three to five of the freshly laid egg capsules were cultured in a beaker containing 800 ml of UV-sterilized seawater until hatching occurred. The seawater in the beaker was exchanged daily and aerated. Water temperature was 15°–17°C and light conditions were 12L:12D throughout the incubation period.

Juveniles used for the microscopic observations of the later stages were raised in a flowing seawater system at the Monterey Bay Aquarium to the maximum date of 129 days post-hatching by methods previously described (Chen *et al.*, 1996). Larger juveniles (1-cm mantle length) were transferred into 180-gallon flowing seawater tanks. Juveniles older than 60 days were fed Selco-enriched adult brine shrimp (*Artemia*), a local species of mysid (*Acanthomysis sculpta*), and guppies (*Poecilia reticulata*) of appropriate size.

Microscopic observations

For light microscopy of newly hatched juveniles, whole hatchlings were fixed in glutaraldehyde (2% in sterile seawater for overnight at 4°C), treated with osmium tetroxide (1% adjusted to oceanic salinity/pH for 1 h), dehydrated in a graded series of acetone (20%–100% in 10% steps), and embedded in Spurr's plastic (Spurr, 1969). Blocks were sectioned transversely in 1-μm intervals and stained with toluidine blue (1 mg toluidine blue/100 ml distilled water, 1–2 mg sodium borate, pH 9.1–9.3).

Older juveniles were fixed in 10% formalin, post-fixed with glutaraldehyde, and embedded in Spurr's plastic as above. For older juveniles too large for serial sectioning, a small section of visceral tissue that included the region of the AN gland was dissected under a stereoscopic microscope and then fixed with glutaraldehyde. So that fe-

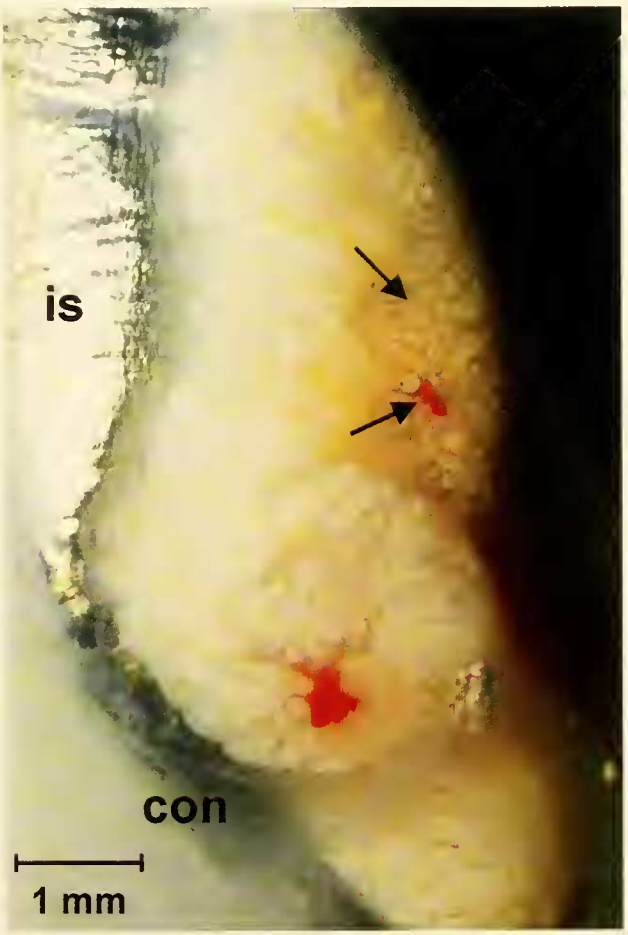

Figure 1. Light micrograph of adult female accessory nidamental gland and associated ink sac. Arrows indicate differential coloring of tubules, which may reflect different levels of colonization by pigmented bacteria. The ink sac (is) and connective tissue (con) located adjacent to the gland are labeled. The second lobe of this bilobed organ can be seen in the lower right portion of the photograph. Magnification bar = 1 mm.

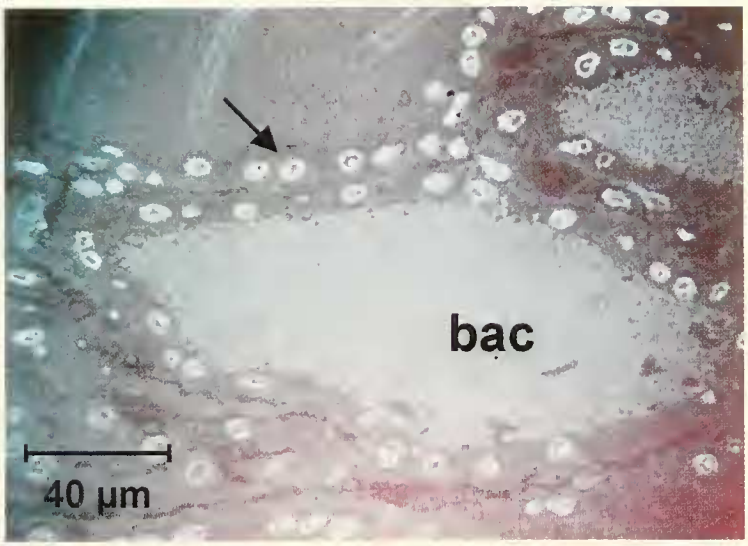

Figure 2. Fluorescent micrograph of 1-μm section of fixed and DAPI-stained tissues of the accessory nidamental gland. Bacteria (bac) densely populate the tubule, which is composed of columnar epithelial cells indicated by the arrow. Magnification bar = 40 μm.

males could be selected, the gonad at the anterior end of the mantle cavity was first examined in thin sections by light microscopy; if it was judged to be an ovary, the sectioning was continued until the region of accessory nidamental gland was observed. The following juveniles were examined (age post-hatching followed by number of specimens in parentheses): 0 days (10); 46 days (1); 75 days (2); 87 days (1); 100 days (2); 129 days (1). Accessory nidamental glands from adult females were dissected and fixed in glutaraldehyde prior to embedding in either Spurr's resin as described above or LR White resin as described previously (Biggs and Epel, 1991).

Transmission electron microscopy

Freshly dissected adult AN gland tissue was cut into 2-mm sections and immediately placed into fixative containing 4 ml 50% gluteraldehyde, 2.38 g HEPES (1 m*M*), and 80 ml sterile seawater (pH 7.2) and incubated on a rotator overnight at 4°C. Fixed glands were rinsed in sterile seawater and post-fixed in osmium tetroxide (1%) for 1 h at room temperature. After two seawater rinses (5 min each), glands were dehydrated in an acetone series (20% to 70%, 10 min each) and held overnight in 70% acetone at 4°C. The acetone dehydration was continued (70% to 100%) using acetone dehydrated with $CuSO_4$. Spurr's resin was measured volumetrically and degassed under a vacuum. Glands were incubated in a 50:50 mix of acetone:Spurr's for 2 h at room temperature, 25:75 mix for 2 h, and 100% Spurr's overnight. Samples were then suspended in fresh Spurr's, placed into BEEM embedding capsules, and polymerized by baking at 70°C overnight. A Porter-Bloom MT2B microtome was used to cut 1-μm sections, which were floated onto Formvar and carbon-coated copper grids (Polysciences). After staining with uranyl acetate and lead citrate, the sections were viewed on a Phillips 201 transmission electron microscope operating at 80 kV.

Results

In the adult female squid, the AN gland is adjacent to the ink sac and digestive organs on the ventral side of the mantle cavity (Fig. 1). The mature gland is composed of thousands of tubules, many of which are heavily colonized by bacteria at the time of spawning (Fig. 2; also see Bloodgood, 1977). Densities of the pigmented bacteria varied within individual tubules, perhaps contributing to the array of colors from orange to dark red displayed on the gland surface (Fig. 1).

To study the mode of infection following hatching, the region near the ink sac and gut tissues was observed in newly hatched juveniles. The gonads were undifferentiated, making it impossible to define the sex of these juveniles, so 10 specimens were analyzed to ensure that some percentage of the sample would be female. In the 1-day post-hatching specimens, no structure indicative of the AN gland could be distinguished (Fig. 3). Only a large ink sac surrounded with muscular tissue and the digestive organ were observed in all the juveniles analyzed.

In the older juveniles (46- to 129-days post-hatching) cultured at the Monterey Bay Aquarium, it was possible

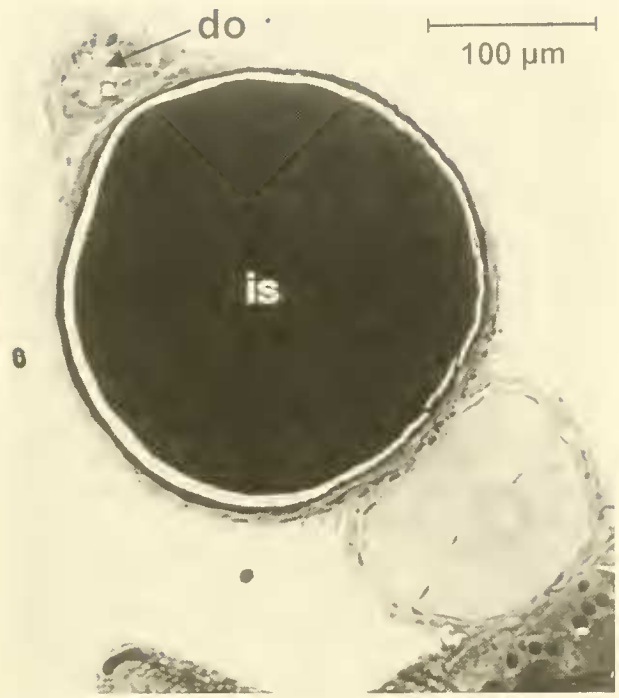

Figure 3. Light micrograph of 1-μm section of 1-day post-hatching *Loligo opalescens* showing the visceral mass, including the ink sac (is) and digestive organ (do). Magnification bar = 100 μm.

to track development of the AN gland. In these specimens, the gonads had differentiated and the sex of the individuals could be determined. In the 46- and 75-day-old female juveniles, the ink sac and digestive organ increased in size, but no AN gland was observed (data not shown). In the 87-day-old juvenile, an early developmental stage of the AN gland was seen flanking the ink sac (Fig. 4). At this stage the gland was a paired structure attached to the outer muscular wall of the ink sac (Fig. 4A) and consisted of a single layer of epithelial cells (Fig. 4B).

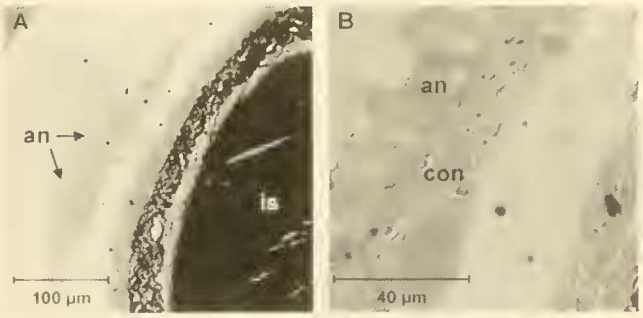

Figure 4. Light micrographs of 1-μm sections of accessory nidamental (AN) gland of 87-day-old juvenile. (A) Gland (an) and associated ink sac (is). Magnification bar = 100 μm. (B) Early tissues of AN gland (an), including the basal connective tissues (con). Magnification bar = 40 μm.

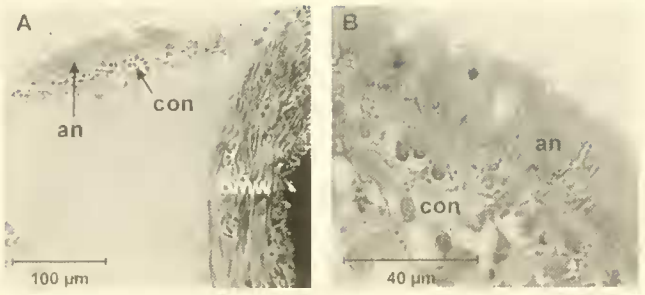

Figure 5. Light micrographs of 1-μm sections of epithelial cells of the accessory nidamental (AN) gland in 100-day-old juvenile. (A) View highlighting gland attachment to the outer muscular wall (omw) of the ink sac. Magnification bar = 100 μm. (B) Cells of AN gland (an) organizing into tubules atop differentiating connective tissues (con). Magnification bar = 40 μm.

No bacteria were evident on the surface of the 87-day AN gland epithelial cells. Between each epithelial cell, typical cell junction complexes were seen and a relatively undifferentiated connective tissue layer was in contact with the basal surface of the cells (Fig. 4B). By 100 days, differentiation of connective tissue pushed the epithelial cell layer towards the mantle cavity. A second layer of epithelial cells was observed, and cilia were occasionally seen on the apical surface (Fig. 5A, B). No bacteria were yet observed in association with the tissue of the AN gland.

In the 129-day-old juvenile, the cell layers of the AN gland were deeply invaginated, forming the primordial tubules (Fig. 6A). In many of these tubules, the end of the structure remained open to the mantle cavity (Fig.

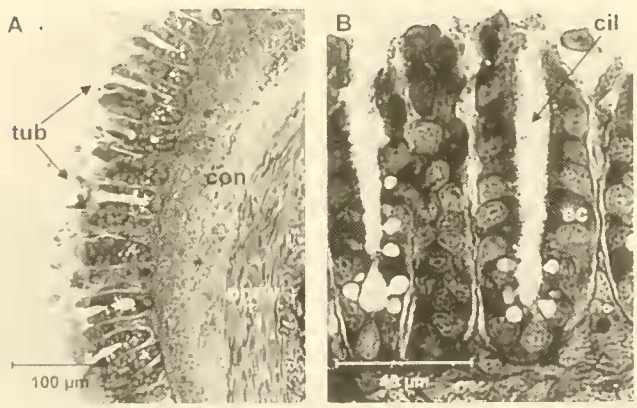

Figure 6. Light micrographs of 1-μm sections of tubules of accessory nidamental (AN) gland in 129-day juvenile. (A) Arrows indicate tubules (tub) open to the mantle cavity. Underlying connective tissue (con) is indicated as well as the outer muscular wall (omw) of the ink sac. Magnification bar = 100 μm. (B) High magnification of tubule structure open to the mantle cavity. Arrow points to cilia (cil) protruding into the tubule. Squid epithelial cells (ec) line the tubule. Magnification bar = 40 μm.

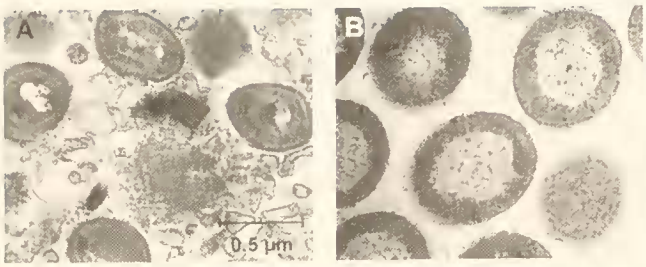

Figure 7. Transmission electron micrographs of bacteria present in accessory nidamental gland at (A) 129 days and (B) in the adult female. Note difference in morphological types and sizes between the juvenile and adult organisms. Magnification bar = 0.5 μm.

6B). Many cilia and some microvilli were observed on the inner surface of both the completely formed and the unformed tubules. The connective tissue layer on the basal surface also continued to undergo significant differentiation. Electron micrographs showed that some of the tubules contained a few bacteria, but these were dissimilar in ultrastructure to the bacteria seen in feral squid from Monterey Bay (in Fig. 7, compare A with B). The bacteria seen in the 129-day specimen have a distinct, gram-positive cell wall; in contrast, organisms found in both the adult gland and purified culture have a gram-negative double membrane. Protozoa were also observed in the lumen of the 129-day AN gland (data not shown). Because the tubule was not completely formed, the presence of the bacteria and protozoa might be adventitious and, therefore, is not evidence for specific infection of the differentiated gland in the 129-day-old squid.

The structures of the epithelial cell components of the AN gland are distinctly different in the 129-day-old squid and the colonized tissue of the adult. The cilia in the juvenile AN gland are more numerous, presumably to facilitate water flow and bacterial colonization (Fig. 8A). In the adult gland laden with microbes, the primary components of the cells are microvilli, with 7- to 10-fold fewer cilia than in the uninfected squid (Fig. 8B).

Micrographs also revealed "secretory vesicles" within the lumen of the mature AN gland (Fig. 9). These globular structures were seen in a layer between the surface microvilli and the bacteria in the center portion of the tubule. The composition and role of these vesicles has not been resolved, but their ubiquity in the adult gland suggests that the AN gland likely contributes both bacteria and structural material to the egg capsule sheath.

Discussion

In the *L. opalescens* egg case, the most probable mode of transmission of bacteria to the female accessory nidamental gland might intuitively appear to be vertical transmission, in which the juveniles encounter the bacteria upon hatching. However, by showing that the AN gland is not differentiated until about 11 weeks post-hatching, this study provides direct evidence that there is no vertical transmission of microorganisms to early hatchlings. Horizontal transmission to juveniles thus most likely occurs from the water column at a later point in development. This time is significantly after hatching: distinct morpho-

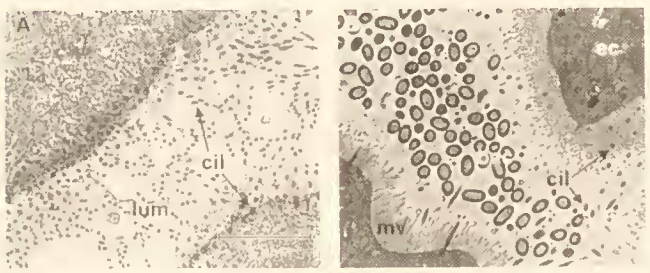

Figure 8. Transmission electron micrographs showing morphology of lumen of accessory nidamental gland at two stages of development. (A) At 129 days, vast numbers of cilia (cil) in the lumen (lum) are surrounded by squid epithelial tissue (ec). (B) In the sexually mature female, microvilli (mv) outnumber the cilia and a dense colony of symbiotic bacteria (bac) resides in the lumen. Magnification bar = 5 μm.

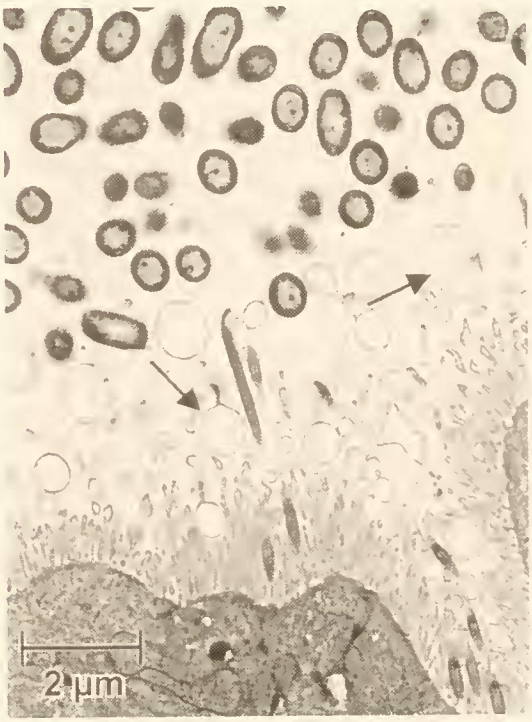

Figure 9. Electron micrograph of "secretory vesicles" at the cell surface of the epithelial cells of the accessory nidamental gland. Arrows designate two examples of this secreted material. Magnification bar = 2 μm.

logical differentiation of epithelial cells is first visible at about 60 days, progressing at 129 days to the formation of tubules similar to those seen in adult squid (see schematic in Fig. 10).

Our analysis of hatchlings and juveniles indicates that a primordial, or rudimentary, AN gland first appears between 75 and 87 days post-hatching. By 129 days post-hatching, tubules resemble the adult morphology and contain numerous cilia (see Fig. 10).

The differentiated AN gland in the 129-day-old female contained small numbers of bacteria, but they bore no ultrastructural relationship to the bacteria seen in adult females brought in from Monterey Bay (Fig. 7). Protozoa were also present in the gland of the 129-day-old female (data not shown), suggesting that the gland was open to the seawater and that a variety of microorganisms could get trapped within the tissue. It is probable that specific infection occurs much later, as the squid approaches sexual maturity. Another possibility is that the appropriate species of bacteria were not present in the artificial conditions of husbandry in the laboratory. It is unknown whether the tubules of the adult gland remain exposed to circulating seawater or close at one or both ends during later gland development. One possibility that remains to be investigated is that the bacteria are acquired at hatching and harbored in an as-yet-unidentified site within the squid hatchling to serve as a reservoir for seeding the developing AN gland.

Bacterial-squid symbiosis has been well studied in the luminous light organ of several cephalopod species (Boletzky, 1970; Herring *et al.*, 1981; McFall-Ngai and Montgomery, 1990; Pringgenies and Jorgensen, 1994). In the case of the sepiolid squid *Euprymna scolopes*, the symbiotic bacteria necessary for luminescence are acquired from the surrounding seawater within hours of hatching (McFall-Ngai and Ruby, 1991).

Although bacterial transmission is horizontal in both the AN gland and the light organ, the time of bacterial colonization differs greatly. This difference is consistent with the different roles for the microorganisms in the

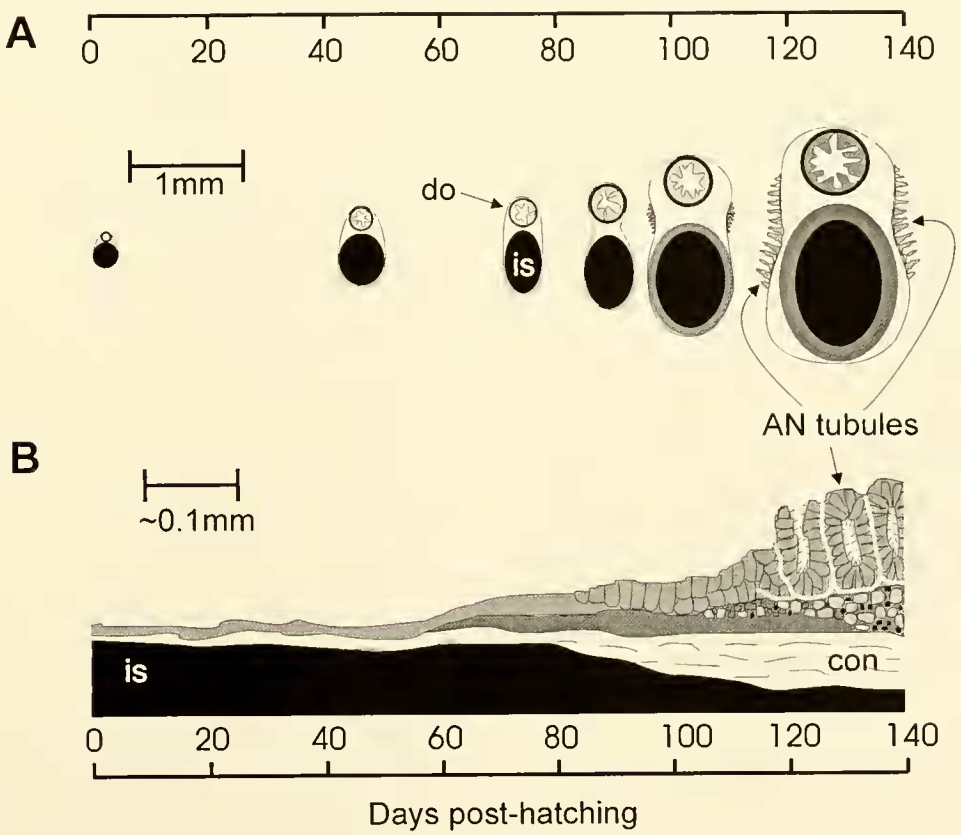

Figure 10. Development of accessory nidamental gland in *Loligo opalescens*. Numbered bars indicate days post hatching. (A) Transverse sections showing the locations of the digestive organ (do) and the ink sac (is) over time are illustrated schematically. (B) Composite drawing, based on light micrographs, showing differentiation and development of gland tissue and associated connective tissue layers. Locations of the ink sac (is) and connective tissue (con) are indicated.

two tissues. The symbiotic bacteria in the light organ of *E. scolopes* provide counterillumination (McFall-Ngai and Montgomery, 1990), which is advantageous just after hatching, when juveniles are especially vulnerable to their predators. In contrast, the role of the AN gland and its associated bacteria is apparently related to sexual maturation and reproduction, which occurs at 1–2 years of age.

Our electron micrographs of tissue from the juvenile and adult AN gland show differences that are remarkably similar to the tissue changes necessary for colonization of *E. scolopes* by *Vibrio fischeri*, in which a cilia-rich epithelium is replaced by a microvilli-rich one (Montgomery and McFall-Ngai, 1993). In the case of the *L. opalescens* AN gland, primary colonization by the squid symbiont probably requires regression of the cilia-rich cells and proliferation of the microvilli-expressing cells. These changes are likely to facilitate bacterial colonization. Preceding infection, the cilia most likely wash copious quantities of seawater through the tubules to "pan" for the specific squid endosymbiont (Ruby, 1996). It is interesting that the squid light organ has been postulated to have its evolutionary origin in the AN gland (Buchner, 1965; Bloodgood, 1977; Montgomery and McFall-Ngai, 1993).

The role of the secretions of the AN gland tissue is still unknown. Proof that the gland contributes gelatinous materials for egg capsule production (Boletzky, 1986) is lacking. Micrographs of adult glands show "secretory vesicles" (Fig. 9) that may be either gland secretions or lipids (Lum-Kong, 1992). It has also been suggested that the AN gland functions in hormonal control of female sexual maturation or mediates reproductive behavior by secreting a pheromone that triggers mating (Richard *et al.*, 1979; Bloodgood, 1977; Lum-Kong and Hastings, 1992).

We suggest that a major role of the AN gland may be cultivating bacteria for deposition to the egg capsules. The precise function of the bacteria is unknown, but the speculation first raised by Biggs and Epel (1991) that microbes provide protective products to the embryos is under investigation and continues to be an attractive hypothesis.

Acknowledgments

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